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Advantages of and Problems with Short-Term Mutagenicity Tests for the Assessment of Mutagenic and Carcinogenic Risk

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The Salmonella microsomal assay has become an indispensible tool for the screening of mutagens and carcinogens, particularly when a large number of samples have to be tested, as in the present context for the screening of air pollution. However, for a more definite identification of potential carcinogens, a verification of the results from bacterial tests has to be performed with a battery of other tests, including point mutations and chromosomal aberrations in eukoaryotic systems.

While there is a close qualitative correlation between the mutagenic and carcinogenic property of chemicals, a corresponding quantitative correlation between the mutagenic and carcinogenic potency is not always found. One reason for this lack of quantitative correlation presumably depends on the fact that cancer is induced in two steps, of which only the initiating, but not the promoting, step constitutes a mutational event, which is reflected by mutagenicity tests.

Present mutagenicity tests have concentrated on discrete major mutations, while mutations of polygenes, acting on quantitative characters, have largely been omitted. Mutational data from Drosophila indicate, however, that polygenes mutate at a considerably higher rate than major genes and that they have a comparatively strong effect in heterozygous condition. It seems of great importance to develop appropriate methods to study induced mutations of polygenic systems and to get a better understanding of the properties of these genetic systems and an evaluation of the risk connected with induced mutations in polygenes.

Introduction

An epidemiological approach to detect chemicals in the environment which cause cancer and hereditary defects has serious limitations. Only about 25 chemicals have been recognized as human carcinogens. At the same time, the variation in cancer frequency between human populations indicates that the vast majority of human cancer is induced by environmental factors. When it comes to the detection of hereditary effects of chemicals, the situation is even more questionable. There are no fully established cases of induced hereditary defects transmitted to the offspring in humans.

There is, however, no reason to believe that this meagre result of epidemiological investigations really reflects the actual situation in human populations. It rather is the inevitable result of the immense difficulties inherited in these studies, such as latency periods of decades, a considerable background level of traits under investigation and a multistep process for tumor induction in which combinations of exogenous and endogenous factors may be involved.

It is therefore quite obvious that we have to rely on experimental rather than epidemiological data for the detection of chemical mutagens and carcinogens in our environment. The most reliable experimental data for extrapolation to humans can be expected from mammalian systems, but unfortunately experiments with mammals also have serious limitations. Tumor induction experiments in mice take 2-3 years and cost somewhere around $200,000 for one test. Even a massive research

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effort with animal cancer tests would not be enough for the screening of thousands of chemicals and chemical mixtures in the human environment.

The Foundation for Short-Term Tests

The way out of this dilemma has been the use of short-term mutagenicity tests to detect both carcinogenic and mutagenic chemicals. This technology is based on two assumptions. The first one is that the ultimate cause of cancer can be brought back to some mutational changes in DNA. Although this still is a hypothesis, there is a wealth of data supporting it: Namely, malignancy is essentially an irreversible trait transmitted from one cell generation to the next. Tumors have a monoclonal origin, that is, they emanate from one single cell. At least the vast majority of known carcinogens and mutagens share an electrophilic property and binding to nucleophilic centers in macromolecules including DNA. With few exceptions, carcinogens have been shown to induce mutations. An impairment of DNA repair leads to an increase of cancer. Cell transformation by oncogenic virus implies a change at the DNA level.

The second assumption behind the use of short-term mutagenicity tests is the similarity of the genetic code and protein synthesis between organisms, which would allow extrapolation between species. The foundation for that is firm enough, but in real life extrapolations between species for mutations meets a number of other problems.

The chemical induction of mutations and cancer involves several steps, which have to be taken into account for any testing procedure. Before discussing the actual test systems it may be appropriate to summarize the course of events for mutations and the complications met with.

The problems in chemical induction of mutations can be considered at three levels: the uptake of the chemical; the biotransformation in the organism; the induction of mutations.

The first level—the uptake of the chemical—implies a major problem in the present context with car exhausts and air pollution. The genetically active compounds are largely bound to particles, but there are widely different opinions as to the availability of such particle bound compounds when they are inhaled and are taken up by macrophages.

The second level of complication is indeed a major one and somewhat of a bottleneck in general for extrapolation of experimental data. Most carcinogens require an activation for their carcinogenic effect, mostly by means of the mixed function oxidase system with cytochrome P-450. The capacity for such an activation of promutagens varies considerably between species, tissues and individuals, but the liver usually constitutes the most active tissue in this respect. There is, however, also an intricate balance between activation and subsequent inactivation of reactive metabolites, for instance by conjugation and this balance also varies between tissues.

Even if a mutagenic chemical which enters the body or is formed through biotransformation reacts with DNA, this does not automatically lead to a mutation. The organism has at its disposal a complicated series of repair systems, which functions as a final barrier against unwanted DNA alterations. A deficiency in the repair system may lead to serious defects and cancer, as illustrated by Xeroderma pigmentosum in humans, which is determined by a recessive gene, eliminating excision repair. Also heterozygotes may have a higher cancer incidence than normal. In some cases the localization of tumors can be brought back to the normal variation between tissues in DNA repair. Ethylnitrosourea thus causes tumors in the brain but not the liver, apparently because the liver but not the brain can repair the mutagenic alkylations (*1*).

Finally the interaction of chemicals with the genetic material results in different types of genetic alterations—point mutations, which imply changes at the nucleotide level, and chromosome aberrations. Furthermore, changes in the number of chromosomes can occur by interactions of chemicals with the spindle fiber mechanism. Chemicals acting on the spindle fibers do not have to be strictly mutagenic that is, interacting with DNA.

Short-Term Test Systems

Salmonella Assay

There obviously are several factors which have to be considered when applying short-term tests for the screening of mutagens and carcinogens. Firstly, mutation induction is generally a rare event, no matter what, and a major problem in mutagenicity screening is the resolving power of the test. For point mutations, microorganisms constitute an ideal material from that point of view. Millions of individuals can be scored in a short time. On the other hand, microorganisms do not meet a second requirement—that is, a relevant biotransformation of chemicals. This problem has been tackled successfully by the addition of a mammalian metabolizing system to microorganisms, in most cases Salmonella. The use of Salmonella for mutagenicity tests is based on the

research by Ames on the biochemistry, organization and operation of the histidine locus in Salmonella (*2,3*). The test system rests on the use of mutations in the histidine locus, which implies that the bacteria cannot synthesize histidine and therefore do not grow on ordinary medium lacking histidine. Only those few bacteria which have mutated back to normal will be able to grow, and the number of colonies will reflect such reverse mutations. A series of Salmonella strains were synthesized with defined mutational changes in the histidine locus exchanges of single bases in DNA (base substitutions) and addition or deletion of bases (frame shift). Reverse mutations to histidine independence require the same type of base changes, that is, base substitution and frame shift. By using different strains of bacteria in the testing, one can therefore establish the actual mutagenic mechanism of the test compound.

Other mutations have been introduced in order to increase the sensitivity of the test system. Thus, a large part of error-free repair has been excluded, and also the lipopolysaccharide envelope has been reduced to facilitate the penetration of chemicals into the bacteria and make them more like mammalian cells. Finally, a plasmid has been introduced, which greatly increases the sensitivity to some chemicals by increasing error prone repair. Two strains with this plasmid, TA 98 for frame shift and TA 100 for base substitutions, have become the most extensively used Salmonella strains; as we will see during this symposium, most mutagenicity screening of air and exhaust samples are performed with TA 98 and to some extent TA 100.

The requirement of a high resolving power for mutations has no doubt been fulfilled with Ames Salmonella strains, but the problem of biotransformation still remains. Salmonella and other bacteria do not have the metabolic capacity of mammals. In order to imitate the conditions in the mammalian body one adds a mammalian liver fraction usually from rats. This liver fraction, the S9 mix, contains the microsomal enzymes responsible for the major activation of indirect mutagens and carcinogens. This combination of Salmonella tester strains with a metabolizing liver fraction constitutes what is widely known as the Salmonella microsomal assay or Ames test. The sensitivity and the accuracy of this assay has made it the principle system for mutagenicity testing. One important reason for this is the close qualitative correlation between mutagenicity in Salmonella tests and animal carcinogenicity. Correlations of close to 90% were reported from various investigations (*4–6*). Rinkus and Legator (*7*) have pointed out, however, that certain classes of carcinogens are not detected as efficiently, and in their survey, which included such chemicals, the correlation between mutagenicity and carcinogenicity was only 75%. Among the classes giving a low response in the Ames test are chlorinated organic compounds, such as DDT, PCB, TCDD, chloroform and carbon tetrachloride. It has been suggested by Ames that these compounds act by mechanism other than electrophilic reaction, namely, by means of radical formation, probably through lipid peroxidation (*8*). Ames has recently constructed new strains which react on alteration of A-T base pairs instead of the ordinary change of C-G base pairs (*8*). These strains are sensitive to peroxides and presumably may pick up chemicals acting by radical formation.

Tests on Higher Organisms

Although this Salmonella assay has become a standard procedure for mutagenicity testing, it cannot be used in isolation for risk identification. It must be supplemented with other test systems. There are several reasons for that. Firstly, Salmonella and other microorganisms can only measure point mutations but not chromosome aberrations or changes in chromosome number. Secondly, the reverse mutation system in Salmonella measures specific DNA changes, but not all types of point mutations may be covered. A forward mutation system, that is, mutations from a functional to a nonfunctional condition of a gene, can be expected to cover all possible genetic changes, which can inactivate the genes. Thirdly, although the genetic code and the protein synthesis are universal, the organization of the chromosomes and the regulation of the gene action differ widely between microorganisms and higher organisms. Fourthly, the metabolic system added *in vitro* constitutes a highly simplified system, and among other problems it does not take into account the balance between the activating and deactivating capacity of the metabolism *in vivo*. The liver S9 mix has the tendency to overestimate the activation of procarcinogens and is therefore somewhat "overpessimistic", which may be an advantage, however, for a prescreening procedure.

For a more reliable risk identification of mutagenic and carcinogenic chemicals it is therefore necessary to use a battery of short-term tests. The supply of possible test systems is indeed huge—a recent survey recognized 116 different tests (*9*). However, only a few can be considered sufficiently established for practical purposes. The time does not permit me to discuss these test systems, but I will just mention some important types of tests (*10-12*).

Beside a number of bacterial systems, which are

used as replacement and complement to Salmonella, mammalian cell cultures, including human cells, are probably the most widely used screening systems. The mutation assay with the HGPRT locus, giving rise to resistance to the purine analogs 8-azaguanine or 6-thioguanine, has been the most common system used. Other systems are resistance to ouabain and diphtheria toxin and forward mutations in the TK (thymidine kinase) locus. An important advantage of these cell culture systems, beside the fact that they measure effects in mammalian cells, is the fact that they are based on forward mutations and thus cover a wide spectrum of mutagenic lesions.

A very important aspect of mammalian cell cultures is the fact that they can be used to study several other genotoxic endpoints, chromosome aberrations, nondisjunction, primary DNA damage and neoplastic transformation.

It may be mentioned that many chemicals are specifically toxic to bacteria and this toxicity may totally mask a mutagenic effect. As an example, emission samples from some power plants run on oil and coal studied by Alfheim and Møller were negative in Salmonella, but also highly toxic. Jenssen at our laboratory got clear positive effects, however, on the same material with hamster cells (*13*). In case of bactericidal samples cell cultures often constitute an appropriate substitute to bacterial systems.

It should be pointed out that most mammalian cell cultures used for mutation work have lost most or all of their metabolic capacity and metabolic systems have to be added as for bacteria. As emphasized before, the biotransformation of chemicals is a bottleneck, particularly for *in vitro* tests. The addition of a microsomal fraction of mammalian liver has implied a very important improvement, but it must be realized that this does not mimic the situation in real life. *In vitro* tests can, however, be combined with a more complete and relevant metabolic system. At our laboratory we have developed a useful system with *in vitro* perfusion of rat liver combined with mammalian cells or Salmonella as indicators of mutations (*14,15*).

The problems concerning biotransformation of chemicals can of course to a great extent be solved by using *in vivo* tests on mammals. Unfortunately, such tests are not nearly as efficient experimentally as *in vitro* tests on cells or bacteria and the resolving power for point mutations is very low and the costs high. For chromosome aberrations, however, a large number of cells can be studied from one animal and such *in vivo* test on mammals can therefore be used as a routine protocol. Particularly the formation of micronuclei in bone marrow cells is a very sensitive, rapid and cheap system for measuring chromosome breakage and effects on chromosome distribution (*16*).

For the very important group of mutations in the germ line, giving rise to hereditary effects, mammalian test systems are far from optimal, although such tests can be performed, particularly on mice, by means of specific locus, translocation and dominant lethal tests. One useful test system in this connection is the fruitfly, Drosophila. Being the genetically best mapped and studied higher organism, many sensitive and sophisticated test systems are available. A very important point here is that Drosophila has the mixed function oxygenase system with P-450 for the activation of procarcinogens. This system is furthermore particularly well developed in spermatides, which make male germ cells sensitive and useful for the screening of chemical carcinogens and mutagens (*17*).

Testing Strategies

Several test systems are required for a risk identification of chemicals, but the tests can be combined according to different strategies. There are primarily two different ways of performing such tests, either using a battery of tests more or less simultaneously or else using a sequence of testing, a "tier" system. For certain chemicals, like food additives and drugs, the recognition of a mutagenic and carcinogenic potential has to be done with maximum accuracy already from the beginning, and a battery of tests is required no matter what. When one is dealing with a large number of samples, as is the usual case with industrial chemicals and emission samples, the limited testing facilities make it necessary to perform the tests in successive steps or tiers, starting with the most sensitive system, which usually is the Salmonella assay. A verification of positive results in the first tier is done at a second tier with more sophisticated and more relevant test systems, such as mammalian tests *in vitro* and *in vivo*, Drosophila, etc. A third testing level should aim at a quantification of the genotoxic risk identified in previous tests.

The usefulness of such a sequential testing procedure with a first screening by means of bacterial systems has been shown in numerous instances. Particularly illustrative are the investigations by Sugimura and his group. The bacterial tests constituted the primary tool to recognize the mutagenic potential of pyrolysis products of food items and this lead to the fundamental discovery and structure determination of super mutagenic amino acid pyrolysis products (*18*). Also the first recognition of the carcinogenicity of the in Japan widely used food

preservative AF 2 can be brought back to bacterial tests in Japan (*19*).

It is no coincidence that we will during this conference over and over again deal with bacterial test results. Because of the large scale and the complexity of air pollution, much of the testing remained at the tier 1 level with bacterial tests. However, the broad approach with various test systems by the EPA program constitutes an important exception. It is to be hoped that a wider use of a second tier level of test will take place in the future.

Quantitative Correlation between Mutagenicity and Carcinogenicity

The qualitative correlation between mutagenicity and carcinogenicity makes it possible to identify carcinogenic chemicals with short-term mutagenicity tests. But at some point we have to deal with the quantitative side of the problem; that is, does a high mutation rate of a compound correspond to a high cancer risk? This is a far more complicated problem.

Attempts have been made to study the correlation between the mutagenicity potency in the Salmonella test and the cancer potency in animal tests. The results have not been particularly encouraging. Although a correlation has been found for many chemicals, as in an analysis by Meselson and Russell (*20*), there are many examples of chemicals where little or no correlation seems to exist. As a matter of fact this is what could be expected when one considers the mechanism of induction of tumors. It is generally recognized that cancer is induced in at least two steps: initiation and promotion. The mechanisms for initiation and promotion are clearly different, and only the initiation step seems to involve a mutation event. Mutagenicity tests therefore reflect only the first step in cancer induction. Cancer tests in animals, on the other hand, usually measure complete carcinogenicity of single compounds, which means that the compound acts both as an initiator and promotor. A correlation between mutagenicity and carcinogenicity can only be expected if there is a complete correspondence between the initiating and the promoting ability of the test compounds, and that is most certainly not the case. It is therefore unlikely that mutation and cancer will show any consistent quantitative correlation.

For a reasonably reliable quantification of the cancer risk, one is still largely dependent on animal cancer data, as Bartsch and Tomatis emphasize in their working paper (*22*). But I think it is appropriate to point out that animal cancer data are mostly obtained with single chemicals, which again implies a simplification of the real situation for human populations, which are exposed to complex mixtures of initiating and promoting agents.

Some Future Aspects of Mutagenicity Testing

The short-term tests have indeed developed into very important tools to trace carcinogenic and mutagenic hazards. But it is hoped that this development has not come to an end, and I would like to finish by pointing out some areas, where I think further research is needed.

One such area concerns induced mutations in quantitative characters. There are two aspects of that problem. The ordinary mutation assays, whether of bacteria, fungi, cell cultures or mammals, all take into consideration only that type of mutation which implies a complete inactivation of the gene function. But all the mutations which just cause a decrease of the gene function are not picked up in the test systems, in spite of the fact that such mutations presumably are the most common ones.

The other aspect of quantitative mutagenic effects concerns polygenes, that is many cooperating genes determining one character, as opposed to major genes, which are the ones studied in ordinary mutagenicity tests. Few organisms have been studied in this respect, but data from Drosophila are of interest and relevance in this connection (*21*). Firstly, investigations of viability and fitness in Drosophila have indicated that polygenic mutations are far more common than major mutations. Secondly, the data indicate that the mutation frequency per gene is much higher for polygenes than for major genes—whatever the molecular mechanism of that can be. Thirdly, polygenic mutations tend to have an unexpectedly strong effect in heterozygous condition. All these data point to the possibility that such polygenes constitute a class of genes, which somehow differ from major genes and also respond differently to mutagenic agents. Of particular concern is the fact that traits like mental ability and speed of reaction in humans must largely be determined by polygenes. By restricting our tests to major genes in mutagenicity screening we may in fact be dealing just with the top of an iceberg.

An important aspect of mutagenicity screening for the detection of carcinogenicity concerns the relation between initiation of cancer and mutation. Although the evidence strongly points to the fact

that cancer is initiated by some kind of a mutagenic event, the actual nature of that event is not known—whether it involves point mutations, chromosomal aberrations, transpositions, DNA methylations or possibly other DNA alterations. The frequency of the initiating event in tumor formation is also obscure, particularly because of the multistep nature of cancer induction. The potency between chemicals for induction of mutations and cancer may, however, be of some interest in this connection. For cancer the potency varies in the order of 10^7 (*22*). If tumor induction requires at least two successive steps, initiation and promotion, the initiating step ought to exhibit an even higher variation in potency, as the promoting step must imply a restriction in tumor formation. It is, however, of interest to note that data of Bartsch et al. (*22,23*) rather may indicate a more narrow range of potency for single point mutations than for cancer with a given set of chemicals. If this indeed were to be the case, it must be concluded that the initiation of tumors either involves a large number of loci or else that the mutagenic event is not comparable to point mutations, but occurs at a higher rate.

There have been specific suggestions of other mechanisms for initiation of cancer than "ordinary" mutations, such as an effect on the methylation process involved in cellular differentiation (*24*) and transpositions of DNA segments (*25*). The transposition hypothesis is interesting because of its resemblance to the viral induction of cancer. However, there is no experimental support for transpositions being involved in induced cancer—in fact carcinogenic agents do not seem to affect transpositions neither in prokaryotes, nor in eukaryotes, such as Drosophila (*26,27*).

Concerning the actual interaction with carcinogens and the genetic material Ames has suggested that radical formation may play an essential role in tumor induction, both for initiation and for promotion. He has furthermore suggested that the comparatively low cancer frequency in humans is the result of the fact that man and primates in general possess a unique radical scavenger in uric acid in the blood (*28*). Investigations of the role of radical reactions in carcinogenesis and mutagenesis will probably require the development of new techniques, as exemplified by Ames synthesis of new Salmonella strains which are sensitive to peroxides.

The potentiality of increasing the predictive value of short term tests to human carcinogenicity probably to a large extent rests on future basic research concerning the actual mechanism of initiation and promotion. A better understanding of these processes will greatly pave the way for more relevant test systems and a safer extrapolation to humans.

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